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(54) Method for diagnosing cancer

(57) A method for diagnosing and/or monitoring the development of cancer by analysing the deoxyribonucleic acid (DNA) in blood plasma, and particularly by detecting any gene alterations in cancer cell DNA, e.g. oncogene mutations or deletions, tumour suppressor gene mutations or deletions, or microsatellite alterations.

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METHOD FOR DIAGNOSING CANCERS

The present invention is concerned with a method for diagnosing and/or monitoring the evolution of various types of cancers after a chemotherapeutic treatment or after surgery.

It is well known that the diagnosis and the monitoring of the evolution of cancers is carried out, in addition to the observation and the direct examination of the tumors, by the analysis of biopsy samples, or in the case of blood cancers, an examination of the bone marrow. This implies either a surgical intervention, or an invasive operation of the biopsy type or further a bone medulla aspiration using a needle. Actually, in addition to the unpleasant if not dangerous nature of such methods to the patient, it was found that they could furthermore lack accuracy. In the case of certain leukemic diseases for example, the analysis of the sample of bone marrow taken has not made it possible to identify all the malignant clone varieties.

The purpose of this invention is hence to provide a method for diagnosing cancers which, on the one hand, is more accurate and more reliable and which, on the other hand, is easier to carry out without the need of resorting to invasive tests on the patients.

The method for diagnosing and/or monitoring the evolution of cancers, object of the invention and aimed at achieving the above purposes, includes the analysis of the deoxyribonucleic acid (DNA) contained in the blood plasma.

In fact, it has now been possible to demonstrate that patients suffering from different cancer diseases show increased amounts of DNA in the blood plasma. The method of diagnosis according to the invention is

hence based on the detection of gene mutations in this plasma DNA, the blood plasma being a human material much more easily accessible than biopsy samples of tumors for example. Thus, oncogene mutations are frequently evidenced in numerous types of malignant tumors and, amongst them the mutations of the ras gene are particularly meaningful. However, the method can be applied to any gene modification of the DNA of the carcinomatous cells, such as mutations and deletions of the genes ras, APC, DCC, P53, etc of any oncogene or anti-oncogene gene (tumor suppressing gene) or furthermore modifications in the microsatellites. It has even been found that different mutations of the ras gene detected in the DNA of the blood plasma could be absent in the DNA of peripheral blood cells or in the case of certain patients suffering from leukemia in the DNA of bone marrow, which tends to confirm the greater reliability of the method according to the invention by comparison to known diagnostic methods.

Generally, the method of diagnosis according to the invention consists in extracting the DNA from blood plasma, in purifying and amplifying this DNA, and thereafter in determining gene mutations or deletions therein, in principle in a comparative test, with the blood plasma of a person presumed to be ill and that of another person in good health, as references.

The scope of the present invention extends to any technique for extracting, purifying or amplifying blood plasma DNA; also, any method appropriate for determining gene mutations can be used.

The diagnostic method according to the invention will now be described in more detail with reference to the two following examples :

Example 1 : Diagnosis of the cancer of the colon by detection of mutations of the K-ras gene.

In this first application of the method according to the invention, use was made of the determination of mutations in the codon 12 of the K-ras gene contained in the adenocarcinomata of the colon. These mutations occur generally upon transition from stage I adenoma to stage II adenoma, before the deletion or the mutation of gene P53, i. e. at a relatively early stage of the development of the tumor.

Blood samples (20-30 ml) were taken from 15 patients with a colorectal adenocarcinoma at different stages of development and heparinized, said patients having received at that time no anticancer drug. Thirteen of the 15 patients underwent subsequently surgery to remove the tumor; also, a total of approximately 400 ml of blood were taken from healthy persons, in order to isolate their plasma DNA.

The DNA was extracted from the tumors and from blood cells using well known conventional techniques.

As to the extraction of the DNA from blood plasma, it can be carried out as follows : the plasma is first subjected to a treatment using phenol, ether and chloroform. After dialysis against a SSC buffer (sodium chloride 0.15 M, trisodium citrate 0.015 M), the product is passed through a concanavallin A-Sepharose \circledast column in order to eliminate the polysaccharides and then centrifuged in a Cs_2SO_4 gradient.

The DNA thus extracted and purified (10 to 100 ng) is then subjected to an amplification by PCR of the first exon of the K-ras gene in a volume of 100 μl .

The amplimers were :

5'-GACTGAATATAAACTTGTGGTAGT-3' and

5'-CTATTGGATCATATCGTCC-3'

The amplifications were carried out in a buffer containing 50 mM of KCl, 10 mM of Tris-NaCl pH 0.3, 200 mM of each nucleotide, 1.8 mM of MgCl₂, 0.2 µM of each precursor and 2.5 units of "AmpliTaq" ® DNA polymerase. 35 cycles were carried out for the DNA of the tumors and of the blood cells and 45 cycles for the DNA of the plasma (94°C during 1 min, 59°C during 1.5 min, 72°C during 1 min, the last cycle being extended to 7 min at 72°C).

Concerning the detection of the mutations, it can be carried out by any known appropriate method. In the present example, it was carried out by two different techniques for each sample tested.

(a) Hybridization of the PCR products with oligonucleotide probes which are mutation specific (according to Verlaan de Vries et al, Gene 50, 313-320, 1986) :

The PCR products were placed in equal amounts on "Zeta-probe" ® membranes (Bio-Rad, Hercules, CA) and hybridized with oligonucleotides specific for wild-type or mutant K-ras. The oligonucleotides were labelled with 32-P ddATP (Amersham GB). In order to separate the perfect hybrids from mismatches, the final washing of the membranes was carried out in a solution containing tetramethylammonium chloride 3 M, 50 mM of Tris-HCl at pH 8.0, 0.2 mM of EDTA and 0.1% of SDS, at 58°C during 1 hour.

(b) Amplification by PCR with amplimers specific of point mutations or amplification by PCR for specific alleles (PASA) (according to Sommer et al, Biotechnology 12, 82-87, 1992) :

In this method, which is more sensitive, the DNA is subjected to an amplification by PCR with amplimers complementary to the normal GLY se-

quences or the mutated ALA, VAL, SER, ASP or CYS sequences. These amplimers which are specific for the mutation have 3' terminations complementary to the mutations at the specific point. The enzyme Taq I polymerase (Perkin-Elmer Cetus, SWI) has no exonuclease activity at the 3' terminus and is therefore unable to amplify the DNA if the mismatch of a single base is located at the 3' terminus of the amplimer.

Each PCR was carried out in a volume of 40 µl of a solution containing 50 mM of KCl, 10 mM of Tris-HCl at pH 8.3, 2 mM of each nucleotide, 0.7 mM of MgCl₂, 0.2 mM of each precursor and 1 unit of "AmpliTaq" ® DNA polymerase. Thirty five cycles were carried out (94°C during 1 min, tempering at 55-62°C during 2 min, extension at 72°C during 1 min). The last cycle was extended to 7 min at 72°C. Each reaction was initiated by the "hot-start" technique. The amplimers used were the following :

5'-ACTTGTGGTAGTTGGAGCTGG-3' for the wild-type K-ras (renaturation at 55°C), 5'-ACTTGTGGTAGTTGGAGCTGC-3' for the ALA 12 mutant (renaturation at 62°C), 5'-ACTTGTGGTAGTTGGAGCTGT-3' for the VAL 12 mutant (renaturation at 61°C), 5'-ACTTGTGGTAGTTGGAGCTA-3' for the mutant SER 12 (renaturation at 59°C), 5'-ACTTGTGGTAGTTGGAGCTA-TGA-3' for the mutant ASP 12 (renaturation at 60°C), 5'-ACTTGTGGTAGTTGGAGCTT-3' for the CYS 12 mutant (renaturation at 59°C) and in each case the anti sense amplimer 5'-CTATTGTTGGATCATATCGTCC-3'.

After amplification, the reaction products were analyzed by electrophoresis on a 0.8% polyacrylamide gel.

By using the first technique (a) described above, it was shown that it is not possible to demonstrate the same mutations in the DNA of the

plasma as those detected in the DNA of the excised tumors (GLY to VAL, GYS to ALA); this technique seems to be applicable only if approximately 10% at least of the total DNA exhibits a point mutation. However, the above mutations could be identified in the plasma DNA with the second technique (b) described above; it appears that this technique enables the identification of mutations in a sample of plasma DNA mixed with a 10^4 to 10^5 excess of normal non-mutated DNA. On the other hand, with the same technique, it was not possible to detect the same mutations on the DNA samples from the blood cells.

Finally, all the control samples from healthy persons showed to be negative, i. e. exhibited no mutations of the plasma DNA.

Example 2 : Diagnosis of cancers caused by myeloid disorders, using the detection of mutations of the N-ras gene.

It is well known that a high incidence of N-ras mutations is observed in the DNA of bone marrow of patients affected by the myelodysplasia syndrome (MDS) or by acute myeloblastic leukemia (AML).

20 to 30 ml of blood were taken from ten patients affected by AML or MDS, this blood was collected on heparin and centrifuged on the "Ficoll Hipaque" gradient (Pharmacia, SWE). Also 400 ml of blood were taken from healthy persons. The interphase containing mononuclear cells was collected and used for extracting the DNA of the blood cells. The upper phase was centrifuged at 2500 G during 15 minutes and the supernatant was used for the extraction of plasma DNA. Furthermore, several samples of bone marrow from the same patients were taken for control analyses.

The DNA from the blood cells and from the bone marrow was isolated

by treatment with protease K (Merck GER) in the presence of SDS, followed by a phenol extraction, a precipitation with ethanol and centrifugation in a Cs₂SO₄ gradient. The plasma DNA was extracted as described in example 1.

The DNA (10 - 100 ng) was amplified in a volume of 100 µl. The amplimers used (Oncogen Science, NY, USA) were 5'-GACTGAGTACAAAC-TGGTGG-3' and 5'-CTCTATGGTGGGATCATATT-3' for the first exon of the N-ras gene. The amplifications were carried out in an automatic "Thermo-Cycler 480" (Perkin-Elmer Cetus, SWI) under the same conditions as those of example 1. Each cycle consisted of a denaturation step at 94°C during 1 minute, a renaturation (at 51°C for N-ras) during 1.5 minutes and an extension of one minute at 72°C, with a third extension segment of 5 seconds per cycle. The last cycle was followed by an extension of 7 minutes at 72°C. The products of the amplification (109 ng) were analyzed by electrophoresis on a 0.8% polyacrylamide gel.

The same two techniques of detection of mutations were employed as in example 1. In the second technique (b), the amplimer used for N-ras was 5'-CTGGTGGTGGTTGGAGCAGA-3' for the ASP 12 mutant, 5'-GGTGGTGGTTGGAGCAGGTT-3' for the CYS 13 mutant and 5'-CTCTATGGTGGGATCATATT-3' as the anti sense amplimer.

The results of the analyses obtained make it possible to confirm that the DNA of the diseased patients exhibited one or several mutations of the codon 12 (GLY to CYS or to ASP) or of the codon 13 (GLY to CYS) in the N-ras gene, while none of these mutations could be found neither in the DNA of the blood cells nor even in that of the bone marrow.

Thus, it is clearly apparent from the two illustrative examples

above that the analysis of the DNA of blood plasma can provide a method for diagnosing and monitoring the evolution of a cancerous disease, which is more convenient, less traumatic (simply a sample of the patient's blood needs to be taken) and sometimes more reliable than known methods making use of biopsy samples.

CLAIMS

1. A method for diagnosing and/or monitoring the evolution of cancers including the analysis of the deoxyribonucleic acid (DNA) contained in the blood plasma.
2. A method according to claim 1, including the extraction of the DNA contained in blood plasma, the purification and the amplification of this DNA, followed by the detection of gene mutations in this DNA.
3. A method according to claim 2, in which oncogene mutations or deletions, mutations or deletions of tumor suppressing genes or further gene modifications occurring in the DNA of carcinomatous cells are detected.
4. A method according to claim 3, in which the detection is applied to any oncogene, antioncogene or tumor suppressing gene, for example to the genes APC, ras, DCC or P53, or furthermore to modifications of microsatellites.
5. A method according to one of claims 2 to 4, in which the DNA is amplified by a polymerase chain reaction (hereafter "PCR").
6. A method according to one of claims 2 to 5, in which the detection of gene mutations is carried out by hybridization of the products obtained by PCR with oligonucleotide probes specific of the mutations.

7. A method according to one of claims 2 to 5, in which the detection of gene mutations is carried out by amplification by PCR and the use of amplimers specific of the point mutations.

INTERNATIONAL SEARCH REPORT

Internat'l Application No
PCT/IB 94/00414

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 22456 (TRUSTEES OF DARMOUTH COLLEGE) 11 November 1993 see the whole document ---	1-7
A	US,A,4 871 838 (J.L.BOS ET AL.) 3 October 1989 see column 14, paragraph 2 - column 15, paragraph 1; claims 1-7 -----	6,7

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

22 March 1995

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INTERNATIONAL SEARCH REPORT
Information on patent family members

Intern: Application No
PCT/IB 94/00414

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9322456	11-11-93	CA-A- 2134552	11-11-93
US-A-4871838	03-10-89	NONE	

RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale No
PCT/IB 94/00414

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 6 C12Q1/68

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)
CIB 6 C12Q

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDÉRÉS COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	WO,A,93 22456 (TRUSTEES OF DARMOUTH COLLEGE) 11 Novembre 1993 voir le document en entier ---	1-7
A	US,A,4 871 838 (J.L.BOS ET AL.) 3 Octobre 1989 voir colonne 14, alinéa 2 - colonne 15, alinéa 1; revendications 1-7 -----	6,7

Voir la suite du cadre C pour la fin de la liste des documents

Les documents de familles de brevets sont indiqués en annexe

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Date à laquelle la recherche internationale a été effectivement achevée 22 Mars 1995	Date d'expédition du présent rapport de recherche internationale 04.04.95
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RAPPORT DE RECHERCHE INTERNATIONALE
Renseignements relatifs aux noms de familles de brevets

Demande internationale No
PCT/IB 94/00414

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
WO-A-9322456	11-11-93	CA-A- 2134552	11-11-93
US-A-4871838	03-10-89	AUCUN	